Ubiquinone Deficiency in an Auxotroph of *Escherichia coli* Requiring 4-Hydroxybenzoic Acid

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1. *Escherichia coli* 156:53D2 synthesized ubiquinone only when the growth medium was supplemented with 4-hydroxybenzoate acid. 2. Little or no vitamin K₂ was formed by the mutant under the growth conditions employed, in contrast with wild-type strains. 3. In the mutant ubiquinone deficiency was correlated with low respiration and with low particulate NADH-oxidase and NADH-cytochrome *b*₁-reductase activity. 4. Preincubation of ubiquinone-deficient particles with ubiquinone-30 largely restored the NADH-oxidase and NADH-cytochrome *b*₁-reductase activities. 5. Various NADH-dye-linked reductases which may be associated with NADH dehydrogenase were not affected by the absence of ubiquinone. 6. The succinate-oxidase complex was less affected than the particulate NADH oxidase by ubiquinone deficiency. 7. A pathway for electrons in the NADH-oxidase complex of the auxotroph of *E. coli* is proposed and its relationship to the pathway in the wild-type strain is discussed.

Studies with mammalian tissue suggested that the benzoquinone ring of ubiquinone is derived from phenylamino acids (Bentley, Ramsay, Springer, Dialameh & Olsen, 1961; Olsen *et al.* 1963). More recently, 4-hydroxybenzoate has been recognized as an intermediate in this pathway (Parson & Rudney, 1965*a*; Olsen, Dialameh, Bentley, Springer & Ramsey, 1965). Similar observations on the importance of 4-hydroxybenzoate in ubiquinone formation have been made with several micro-organisms (Parson & Rudney, 1964) and in *Escherichia coli* the biosynthetic pathway probably leads from shikimic acid and chorismic acid to ubiquinone via 4-hydroxybenzoic acid (Cox & Gibson, 1964).

E. coli contains the naphthaquinone, vitamin K₂-40 (Lester & Crane, 1959; Bishop, Pandya & King, 1962), as well as several ubiquinone isoprenalogues of which ubiquinone-40 is the largest component (Friis, Daves & Folkers, 1966). Demethyl-vitamin K₂ has also been reported in this organism (Dolin & Baum, 1965).

An important role for quinones in the respiration of *E. coli* was deduced from the sensitivity of aerobic growth to intense ultraviolet irradiation (Kashket & Brodie, 1962). Studies on electron transport implicated vitamin K₂ in the NADH-oxidase complex and ubiquinone was assigned a role in the succinate-oxidase system (Kashket & Brodie, 1963*a,b*). Ubiquinone may also be a carrier between

formate dehydrogenase and cytochrome *b*₁ in *E. coli* grown with nitrate as terminal acceptor (Itagaki, 1964).

A multiple aromatic mutant, *E. coli* 156, requires 4-hydroxybenzoic acid for rapid aerobic growth in addition to phenylalanine, tyrosine, tryptophan and 4-aminobenzoic acid (Davis, 1952; Jones & Lascelles 1967). This mutant was found to lack ubiquinone when grown without 4-hydroxybenzoic acid and the lack of ubiquinone was correlated with low respiration and low NADH-oxidase activity. The organism offered a method of studying the role of ubiquinone without resort to solvent extraction or ultraviolet irradiation. The present work suggests that in the auxotroph ubiquinone is an important component of the NADH-oxidase chain and is located between the flavoprotein dehydrogenase and cytochrome *b*₁. Some of these results have been reported briefly (Jones & Lascelles, 1966).

METHODS

Organisms. *Escherichia coli* 156:53D2 and *E. coli* W were maintained and grown as described by Jones & Lascelles (1967).

Preparation and fractionation of cell-free extracts. The organisms after harvesting and washing were disrupted by passage through a French pressure cell as described by Jones & Lascelles (1967). The cell-free extract was fractionated into soluble and particulate preparations by centrifuging at 100 000*g* for 80 min. at 0° in a Spinco model

L ultracentrifuge (Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.). Protein contents were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

Measurement of respiration. The rate of oxygen uptake by washed cell suspensions and by extracts was measured polarographically with an oxygen electrode (Rank Bros., Boffersham, Cambridge). The latter was connected to a Beckman 96260 adapter box and continuous strip recorder (Beckman Instruments Inc., Fullerton, Calif., U.S.A.). The electrode was standardized against 3 ml. of 40 mM-phosphate buffer, pH 7.2, saturated with air at 25° and the same buffer with excess of sodium dithionite was added to remove the oxygen.

Determination of quinone content. The organisms after harvesting during exponential growth were washed in 0.9% KCl, suspended in water and freeze-dried in dim light. The dried cells were extracted three times with 5 vol. of ethanol-ether (3:1, v/v) or acetone. The combined extracts, after evaporation, were taken up in acetone, evaporated again and applied in ethanol to activated plates (0.5 mm.) of Kieselgel G (Merck reagent supplied by Camlab Ltd., Cambridge). The plates were developed either in chloroform-light petroleum (b.p. 40–60°) (1:4, v/v) or benzene-chloroform (4:1, v/v), adapted from the system of Wagner, Hörhammer & Dengler (1962).

The areas containing quinones were visualized in ultra-violet light, removed and extracted three times with ethanol. The quantity of each quinone was determined spectrophotometrically with an Optica model CF4DR recording spectrophotometer (Optica Ltd., Gateshead, Co. Durham). Ubiquinone was estimated by the difference in oxidized minus borohydride-reduced extinction at 275 m μ [$E_{1\text{ cm. }1\%}^{\text{ox-red.}}$ at 275 m μ 176 (Lester & Crane, 1959)]. Vitamin K₂ was estimated from the absorption of the purified sample at 249 m μ and the difference in absorption at 249 m μ and 285 m μ was used as a measure of purity [$E_{1\text{ cm. }1\%}$ at 249 m μ , 264 (Bishop *et al.* 1962)].

Determination of haem content. The haem content was estimated spectrophotometrically as described by Porra & Lascelles (1965). Spectra were obtained with an Optica model CE4DR recording spectrophotometer. Haem contents were calculated with $\Delta E_{\text{cm}}^{\text{red-ox}}$ 137 (R. J. Porra, personal communication).

Measurement of enzyme activities. NADH oxidase was measured spectrophotometrically by the decrease in absorption at 340 m μ , dissolved oxygen being used as terminal acceptor, in 40 mM-tris-HCl, pH 7.6, at 25°. Alternative acceptors were added in the following quantities to the 3 ml. reaction cuvette: ubiquinone-0, 0.3 μ mole; potassium ferricyanide, 4 μ mole; menadione, 0.25 μ mole; 2,6-dichlorophenol-indophenol, 0.4 μ mole; cytochrome *c*, 1.2 μ mole.

The NADH-oxidase and succinate-oxidase systems were also measured polarographically with an oxygen electrode with NADH (2 μ mole) and succinate (50 μ mole) added respectively as substrates.

NADH-cytochrome *b*₁ reductase was followed by using an Aminco-Chance dual-wavelength spectrophotometer (Aminco Instrument Co., Silver Spring, Md., U.S.A.) at 559 m μ with the reference beam set at 575 m μ in 33 mM-tris-HCl buffer, pH 7.6.

Succinate dehydrogenase was assayed by the method of

Arrigoni & Singer (1962), with phenazine methosulphate and 2,6-dichlorophenol-indophenol as acceptors. Potassium ferricyanide (1.2 mM) was used as an alternative acceptor in some experiments.

Lipoyl dehydrogenase was determined by the method of Williams (1965), modified by the use of reduced lipoic acid as hydrogen donor.

Chemicals. Ubiquinone and vitamin K₂ isoprenologues were gifts from the British and American subsidiaries of Hoffmann-La Roche Ltd. SN5949* was kindly provided by Dr D. E. Griffiths, University of Warwick. Reduced lipoic acid, HOQNO* and Dicoumarol were from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and cytochrome *c* from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Ethanol and light petroleum (b.p. 40–60°) were redistilled and dried before use.

RESULTS

Quinone and haem contents and respiration of E. coli under various growth conditions

Quinone content. High concentrations of ubiquinone were found in the auxotroph grown with 4-hydroxybenzoic acid but little or no vitamin K₂ was found. The quinone content was not changed by the further addition of 3,4-dihydroxybenzoic acid to the growth medium, although this compound improved growth. Organisms grown without 4-hydroxybenzoic acid, even in the presence of succinate, contained no detectable ubiquinone (Table 1).

Wild-type *E. coli* W contained both vitamin K₂ and ubiquinone when grown aerobically on glucose. Both quinones were also found in wild-type strain *E. coli* 518.

Haem content. The mutant grown without 4-hydroxybenzoic acid is unable to form adequate amounts of succinate for growth (Jones & Lascelles, 1967). In addition to its role in lysine and methionine biosynthesis, succinyl-CoA is a precursor of δ -aminolaevulinic acid, which is the first intermediate in tetrapyrrole formation (see Lascelles, 1964). The total haem content of organisms grown without 4-hydroxybenzoic acid was low compared with that of wild-type cells and the mutant grown with 4-hydroxybenzoic acid. Supplementation of the growth medium with succinate restored the haem content of the ubiquinone-deficient cells to normal (Table 1).

Respiratory activity. *E. coli* 156 grown without 4-hydroxybenzoic acid did not respire appreciably on glucose, succinate, pyruvate, malate and other carbon sources. When succinate was added to the growth medium to ensure a normal haem content, no increase in respiration was observed (Table 1). Organisms grown with 4-hydroxybenzoic acid and

* Abbreviations: SN5949, hydroxy-3-(2-methyl)octyl-2,1,4-naphthaquinone; HOQNO, 2-*n*-hexylhydroxyquinoline *N*-oxide.

Table 1. *Effect of 4-hydroxybenzoate deficiency on quinone and haem content and on respiration*

E. coli strains 156 and W were grown as described in the Methods section with the other additions shown below.

Organism	Additions to medium	Quinone content (μ mole/g. dry wt.)		Haem content (m μ mole/mg. of protein)	Respiration (μ l. of O ₂ /mg. of protein/hr.)		
		Vitamin K ₂	Ubiquinone		Glucose	Succinate	Malate
<i>E. coli</i> W	None	0.09	0.32	0.13	295	115	110
<i>E. coli</i> 156	None	<0.01	<0.005	0.02-0.07	<20	<10	<10
<i>E. coli</i> 156	Sodium succinate (0.1 mM)	<0.01	<0.005	0.16	<20	<10	<10
<i>E. coli</i> 156	4-Hydroxybenzoate (1 μ M)	<0.01	0.7	0.14	115	40	65
<i>E. coli</i> 156	4-Hydroxybenzoate 3,4-Dihydroxybenzoate (1 μ M)	<0.01	0.65	*	140	55	*

* Not tested.

Table 2. *Effect of 4-hydroxybenzoate deficiency on NADH-oxidase activity*

E. coli strains W and 156 were grown as described in the Methods section with other additions as shown below. NADH oxidase was measured spectrophotometrically by the decrease in NADH absorption and is expressed as μ moles of NADH/mg. of protein/hr. at 23-25°.

Organism	Additions to medium	NADH-oxidase activity	
		Super- natant fraction	Parti- culate fraction
<i>E. coli</i> W	None	0.6	60
<i>E. coli</i> 156	None	0.3	2.5
<i>E. coli</i> 156	Sodium succinate (0.1 mM)	0.32	2.8
<i>E. coli</i> 156	4-Hydroxybenzoate (1 μ M)	0.7	20
<i>E. coli</i> 156	4-Hydroxybenzoate 3,4-Dihydroxybenzoate (1 μ M)	0.8	28

therefore containing ubiquinone respired rapidly on these carbon sources. The small stimulation of growth by 3,4-dihydroxybenzoic acid was reflected in a small increase in the respiration of these organisms. Wild-type *E. coli* respired more rapidly than the auxotroph under all the conditions tested.

Ubiquinone in electron transport in E. coli

Effect of growth with and without 4-hydroxybenzoate on NADH-oxidase activity. The NADH-oxidase activity of *E. coli* W and of the mutant grown with 4-hydroxybenzoic acid was found almost entirely in the 100 000 g particulate fraction

Table 3. *Effect of inhibitors on particulate NADH-oxidase activity*

E. coli 156 was grown in glucose-salts medium supplemented as described in the Methods section and with the further addition of sodium succinate (0.1 mM). HOQNO and SN5949 were dissolved in ethanol and a small volume was added to the reaction cuvette. NADH-oxidase activity was measured as described in Table 4. 4-OB, 4-hydroxybenzoic acid (1 μ M).

Inhibitor	Concn. in cuvette	Inhibition of activity (%)			
		<i>E. coli</i> 156			<i>E. coli</i> W
		With 4-OB	Without 4-OB		
Sodium cyanide	1 mM	70	24		72
	2 mM	90	35		85
HOQNO	60 μ M	92	59		85
SN5949	40 μ M	78	45		90
Dicoumarol	0.3 mM	56	26		88

(Table 2). The activity was inhibited by cyanide, HOQNO and the substituted naphthaquinone, SN5949 and less strongly by Dicoumarol (Table 3). Preparations of *E. coli* W, which were consistently of higher specific activity than those of the mutant, were somewhat more sensitive to Dicoumarol.

Only a very low particulate NADH-oxidase activity was found in organisms lacking ubiquinone; this activity was far less sensitive to respiratory inhibitors than that found in preparations from cells containing quinone (Tables 2 and 3).

A low concentration of soluble NADH oxidase was found in both mutant and wild-type organisms and was independent of the presence or absence of ubiquinone. The soluble oxidase was insensitive to cyanide and HOQNO and was stimulated by SN5949.

Table 4. *Reconstitution of particulate NADH-oxidase activity from E. coli 156 grown without 4-hydroxybenzoic acid*

E. coli 156 was grown as described in Table 3 and the particulate NADH-oxidase activity was measured by the decrease in absorption at $340\text{m}\mu$. Quinones, except menadione, were dissolved in ethanol and a small volume ($<0.05\text{ml.}$) was added to the cuvette, which was immediately shaken vigorously to ensure a fine emulsion. Activities with ferricyanide, menadione and cytochrome *c* were measured in the presence of 2mM -sodium cyanide. 4-OB, 4-hydroxybenzoic acid ($1\mu\text{M}$).

Additions to cuvette	Final concn. (mm)	NADH-oxidase activity <i>E. coli</i> 156	
		Without 4-OB	With 4-OB
None		3.6	18.4
Ubiquinone-40	0.07	12.5	*
Ubiquinone-30	0.06	14.8	19.0
Vitamin K ₂ -45	0.1	4.0	*
Vitamin K ₂ -30	0.08	3.8	17.0
Ubiquinone-0	0.1	72.5	46.0
Menadione (vit. K ₃)	0.06	36.0	17.0
Potassium ferricyanide	1.4	44.0	50.5
Cytochrome <i>c</i>	0.4	0.1	6.0

* Not tested

Reconstitution of NADH-oxidase activity. The ability of various quinones to restore the low activity found in particulate preparations of organisms grown without 4-hydroxybenzoic acid was tested. Ubiquinone-30 and -40 caused an almost complete restoration of activity (Table 4) and the reconstituted activity was inhibited by HOQNO and cyanide. Long-chain naphthaquinones were ineffective. Ubiquinone-0 and menadione also increased the activity many times but the activity was not sensitive to HOQNO and cyanide. Other artificial acceptors, which did not contain a quinone ring, 2,6-dichlorophenol-indophenol and ferricyanide, were also capable of stimulating rapid NADH oxidation in preparations either containing or lacking ubiquinone. In contrast, NADH-cytochrome *c* reductase was ubiquinone-dependent indicating that cytochrome *c* accepted electrons from a site beyond ubiquinone in the electron-transport chain.

The oxidase activity of the auxotroph grown with 4-hydroxybenzoic acid was destroyed by ultra-violet irradiation (Brodie, 1963). The activity was partially restored by ubiquinone-30 but not by naphthaquinone isoprenalogues. Similar experiments were carried out with preparations from *E. coli* W, which were not stimulated by ubiquinone but were partially reconstituted by vitamin K₂-30

Fig. 1. Effect of ubiquinone deficiency on NADH-cytochrome *b*₁-reductase activity of *E. coli* 156. The reduction of cytochrome *b*₁ was measured by the difference in absorption between $559\text{m}\mu$ and $575\text{m}\mu$ with (a) particulate preparations of organisms grown with 4-hydroxybenzoate and (b and c) similar preparations of organisms grown without 4-hydroxybenzoate. The reaction mixture contained: tris-HCl buffer, pH 7.6, $100\mu\text{moles}$; KCN, $6\mu\text{moles}$; 1.8mg. of protein of particles; water to 3ml. NADH, $0.8\mu\text{mole}$, was added as shown. (c) Particles from cells grown without 4-hydroxybenzoate were preincubated with ubiquinone-30, $0.12\mu\text{mole}$, in 0.03ml. of ethanol.

as has been previously found by Kashket & Brodie (1963b). These results suggest that ubiquinone is a component of the NADH-oxidase chain of the auxotroph when grown with 4-hydroxybenzoate, but this does not appear to be so with the wild-type strain.

NADH-cytochrome b₁ reductase. Although the results implicate ubiquinone in the electron-transport chain of the auxotroph, they do not show the location of the quinone site. To obtain evidence on this point the NADH-dependent reduction of cytochrome *b* in particulate preparations of *E. coli* was measured in an Aminco-Chance dual-wavelength spectrophotometer. The rapid reduction of the cytochrome by NADH was observed with particulate preparations of the auxotroph grown with 4-hydroxybenzoic acid. In contrast only a slow and limited reduction was found with particles from ubiquinone-deficient organisms; this activity

Table 5. *Effect of 4-hydroxybenzoate-deficiency on various succinate-linked enzymic activities*

E. coli 156 was grown as described in Table 3. The succinate-oxidase system was measured polarographically and the dehydrogenase activities were followed spectrophotometrically as described in the Methods section.

Addition to medium	Succinate oxidation linked to		
	Phenazine metho-sulphate*	Potassium ferri-cyanide*	Oxygen†
None	0.65	0.25	15
4-Hydroxybenzoic acid (1 μ M)	0.7	0.45	30

* μ moles of succinate/mg. of protein/hr. at 22°.

† μ l./mg. of protein/hr. at 35°.

was greatly increased by preincubation of the particles with ubiquinone-30 (Fig. 1).

Effect of growth without 4-hydroxybenzoic acid on the activity of succinate-dehydrogenase complex. Little succinate-oxidase activity was found in particulate preparations of *E. coli* W or *E. coli* 156 unless the preparation was supplemented with supernatant fraction. The succinate-oxidase activity of unfractionated extracts of *E. coli* 156 grown without 4-hydroxybenzoic acid was approximately half that of organisms grown with the compound. The succinate-ferricyanide-reductase activity was also decreased by about half whereas the dehydrogenase activity measured with phenazine methosulphate as primary acceptor was not affected (Table 5). The succinate-oxidase activities of extracts of *E. coli* W and *E. coli* 156 were all inhibited by sodium cyanide, HOQNO and SN5949.

DISCUSSION

The inability of the multiple aromatic mutant of *E. coli* to form ubiquinone in the absence of 4-hydroxybenzoate is further evidence of its role in ubiquinone biosynthesis. In the absence of 4-hydroxybenzoate a small growth response to ubiquinone could be detected with the auxotroph (R. G. W. Jones & J. Lascelles, unpublished observations). The enzymic steps between 4-hydroxybenzoate and ubiquinone are not known although 2-decaprenyl phenol is accumulated in dark, anaerobic cultures of *Rhodospirillum rubrum* and may be an intermediate (Parson & Rudney, 1965b; R. K. Olsen *et al.* 1965). The biosynthesis of the naphthaquinone ring of vitamin K also involves the shikimic acid pathway and Cox & Gibson (1966) have shown that the incorporation of [14 C]shikimic acid into vitamin K₂ in suspensions of *E. coli* is

depressed by 3,4-dihydroxybenzaldehyde. However, in the present study it has not been possible to show the synthesis of vitamin K₂ in the auxotroph.

By using the ability to grow the auxotroph with and without 4-hydroxybenzoic acid, a positive correlation was found between ubiquinone content, respiration and rapid aerobic growth. The low haem content of these cells cannot be a major factor as the addition of succinate to the growth medium restored the haem content to normal but caused only a limited improvement in growth with no increase in respiration.

The role of ubiquinone in respiration was studied by investigating the succinate-oxidase and particulate NADH-oxidase complexes. The sensitivity of these activities in preparations of *E. coli* W and the auxotroph containing ubiquinone to known respiratory inhibitors suggests a pathway involving cytochrome *b*₁ and a terminal oxidase (Jackson & Lightbown, 1958; Estabrook, 1958). The latter is probably the carbon monoxide-binding pigment, cytochrome *o*, described by Castor & Chance (1959). The experimental results strongly implicate ubiquinone in the NADH-oxidase complex of the auxotroph although equivocal results were obtained with the succinate-oxidase complex. Since ubiquinone-30 is capable of largely restoring the NADH-oxidase activity of ubiquinone-deficient preparations and the characteristic inhibitor sensitivity, the other enzymes and cofactors are probably present in the deficient organisms.

The low NADH-cytochrome *b*₁-reductase activity of ubiquinone-deficient particles indicates that the quinone site precedes cytochrome *b*₁. The rapid oxidation of NADH in the presence of some artificial acceptors in preparations of cells grown with and without 4-hydroxybenzoic acid suggests that the primary flavoprotein dehydrogenase is always present (Machinist & Singer, 1965). The assays with artificial acceptors are not specific for the primary dehydrogenase as lipoic acid is also active with these acceptors (Massey, 1962). In the present work it was not possible to dissociate entirely the activity due to the primary dehydrogenase from lipoyl dehydrogenase. However, neither NADH nor lipoyl dehydrogenases were affected by ubiquinone deficiency (Jones, 1966). The observations indicate that ubiquinone occupies a site between the NADH dehydrogenase and cytochrome *b*₁ in the NADH-oxidase complex of the auxotroph.

Kashket & Brodie (1963b) found that vitamin K₂ was associated with the NADH-oxidase complex of *E. coli* W. Considerable differences were found in the behaviour of the auxotroph and of *E. coli* W in the present work and observations with the latter organisms do not conflict with the results of Kashket & Brodie. It may be that the two quinones may fulfil a similar function in different strains or,

possibly, under different growth conditions. Several studies have indicated substantial differences in the quinone content of closely related strains of bacteria (Gibbons & Engle, 1965; Knowles & Redfearn, 1966).

Many examples are now available of quinones acting as carriers in bacterial electron-transfer systems (see Brodie, 1965). In many of these examples, including the present work, the quinone acts as a carrier between the flavoprotein dehydrogenase and the cytochrome chain (Francis & Phizackerley, 1965; Knowles & Redfearn, 1966; Downey, 1964; Itagaki, 1964; Brodie, 1965). An exception to this pattern has been reported in *Corynebacterium diphtheriae*, where vitamin K₂ may act between cytochrome *b* and *c* (Krogstad & Howland, 1966).

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